Declaration of Randolph S. Porubcan re
US Patent Application Serial No. 10/74 ,402

I, Randolph S. Porubcan, understand that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

I. Experience and Education

I received a Masters degree in Biochemistry from the University of Wisconsin in 1975. I have worked extensively with probiotic microorganisms, including in the design and building of construction facilities, and in the production and sale of agriculture-related probiotics, since the 1980s. I am currently the CEO of Therabiotics, Inc., which develops a new generation of probiotics and related products. I am a named inventor of several patents in the probiotics field.

II. Where Calcium Alginate Is Present, the Claimed Formulation Is not Present

In addition to the material in Application Serial No. 10/743,40 , I note that the Examiner, in a recent Office Action (dated June 1, 2005) stated: "there is nothing on record to show (via a side-by-side comparison) that the formulation of McGrath et al. is not the same [as that claimed] and would not inherently have the same water activity." The important feature set forth in the claims which is not disclosed in McGrath is that in McGrath et al., the probiotic bacteria in the feed is not intended to be acid-resistant, and acid resistance is not mentioned. McGrath et al. note that the mixture includes alginate, and: "Preferably, the alginate is an alkaline earth metal alginate, and most preferably the alginate is a calcium or barium alginate." (para. 23). The experiments below demonstrate that calcium alginate, in a formulation with microcrystalline cellulose, bacteria and grape skin extract, do not form "an alginic acid gel is formed which shields the probiotic bacteria from the antibiotic effects of the acidic environment" on exposure to an acidic environment, as required in claim 1. It is clear, therefore, that the McGrath et al. formulation, containing calcium alginate, does not form an alginic acid gel. Moreover, although as described in para. 47 of McGrath et al., sodium alginate can be used in formulating the McGrath et al. mixture, thereafter:

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> The suspension [including bacterial cells and sodium alginate] is then added to a solution containing calcium ions to cause a gel to form. Alternatively, the cells may be suspended in the calcium-containing solution and added to a soluble alginate solution. The gel comprises calcium alginate, and contains immobilised probiotic cells suspended in the alginate.

However, when sodium alginate is mixed with a "calcium-containing solution," a gel-like insoluble material forms – but an *alginic acid gel* does not form on contact with an acidic environment, due to the presence of the calcium ions.

III. Experimental; Results; Conclusions

Preparation Method

1) Calcium alginate was prepared by adding aqueous one molar calcium chloride to a solution containing 4% weight/volume sodium alginate (Sigma-Aldrich Cat. # A7128) while constantly agitating the solution magnetically. The insoluble precipitate of calcium alginate that formed was filtered out on Whatman #1 filter paper, rinsed with distilled water, and vacuum dried to 5 % moisture content.
2) A dry blend was prepared in a lab-scale double-cone mixer with all ingredients added by weight percent: 20% calcium alginate from Step 1 above, 63% microcrystalline cellulose (Avicel PH112, FMC Biopolymer), 10% freeze-dried *Lactobacillus paracasei* strain F-19 (200 billion cfu/gram, Medipharm, Inc.), 5% grape skin extract (AC 12z WSP, Chr. Hansen, Inc.), 2% silica (Syloid 63, W.R. Grace & Co.). The ingredients were blended at 60 rpm for 10 minutes until uniformly lavender in color from the grape skin extract. The resulting powder was hand filled into 50 size "0" cellulose capsules (HPMC Vcaps, Capsugel) at 325 mg per capsule. The resulting powder, prior to filling the capsules, had a water activity of 0.050 measured on a Rotronic Model A2 Hygromer. All operations were carried out in a low humidity room (20% relative humidity). The resulting capsules were stored in amber glass bottles at 25 C, tightly closed, with silica gel packets for moisture absorption, until further tested.

Testing Method (Stability in pH 1.6 Simulated Gastric Juice)

1) Ten capsules were submersed in pH 1.6 simulated gastric juice (2.0 g sodium chloride + 3.2 g pepsin + 7.0 ml HCl + distilled water to make 1 liter) for 90 minutes. After 90 minutes the capsule contents (sausage shaped gel-structures or loose, non-structured flocculant forms) were rinsed with distilled water and partially dried on a paper towel for 15 minutes.
2) The quality index of the resulting gel-structures were rated on a scale of 1 to 5: 1 = Little or no surviving gel-structure, capsule content mostly spilled out into gastric juice; 2 = Poor quality gel-structure with minimal sausage shape and internal contents largely exposed to gastric juice; 3 = Satisfactory sausage shaped gel-structure with some penetration of gastric juice into internal contents, burgundy-red outside, dark purple with some wetness inside; 4 = Good quality sausage shaped gel-structure with some swelling, intact and dry to semi-dry inside, burgundy-red outside, lavender colored inside; 5 = Excellent sausage

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shaped gel-structure, completely intact and dry inside, burgundy-red outside, lavender inside.

3) The viable plate count and pH of the contents of the gel-structures were determined after the 15 minute drying time (note: this determination is reliable for gel-structures having a quality index of 3 or better, for gel-structures with a quality index below 3 only an approximate analysis can be performed). Plate counts were made on MRS agar (Oxoid, CM 361) and incubated in anaerobic jars for 72 hours at 37 C, all colonies were counted; pHs were determined with an Orion pH meter by mixing the gel-structure 1:1 with distilled water and immersing the pH electrode into the mixture.

<u>Results – Stability in pH 1.6 Gastric Juice</u>

1) Quality index (sausage gel-structure): All ten capsules came apart and dissociated without any resulting sausage structure, yielding a quality index of 1. The contents of all 10 capsules spilled out into the gastric juice and formed a shapeless floc that was bright burgundy-red in color (indicating that the contents were uniformly exposed to the pH 1.6 environment).
2) The pH determinations yielded an average pH of 1.9 for the internal contents of the 10 capsules.
3) The plate count determinations yielded an average cfu of < 10^5/g (less than one tenth of a million cfu/g) for the internal contents indicating a loss of over 99.99% in viability for the *Lactobacillus paracasei* strain F-19. [Note: the initial concentration of *L. paracasei* in the capsules was 20 billion cfu or 20 x 10^9/g]

<u>Conclusions</u>

1) The use of calcium alginate in this formulation did not provide any significant protection for the *L. paracasei*, probiotic culture to exposure to pH 1.6 gastric juice.
2) The use of calcium alginate did not result in the formation of a sausage shaped gel-structure required for protection from low pH gastric juice.
3) The extent of reduction in viability (>99.99%) of the *L. paracasei*, probiotic culture makes the use of calcium alginate commercially unacceptable in the tested formulation.
4) As seen in the Patent Application 10/743,402, Examples 3 to 8, the use of sodium alginate in a similar formulation tested in pH 1.6 gastric juice did provide significant protection from the gastric juice, as evidenced by no apparent reduction in viability of the culture in such capsules.

I make the foregoing statements having understood that willful false statements and the like are punishable by fine or imprisonment, or both, and may jeopardize the validity of the application or any patent issuing thereon.

[signature]

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